On page 1, line 24, please insert –lymph– before "node".

On page 2, line 14, please insert –Early Breast Cancer Trialistic Collaborative Group-- before "1992" in the left parenthesis.

On page 2, line 14, please insert –The Ludwig Breast Cancer Study Group-- before "1989".

At page 5, line 24, please replace "Figures 1A through 1C" with -- Figures 1A, 1B and 1C--.

At page 6, line 4, please replace "Figure 3" with -- Figures 3A and 3B--.

At page 6, line 9, please add -- Figure 3A-- after "(GE-5".

At page 6, line 9, please add -- Figure 3B-- after "(LE-6".

On page 7, line 16, please delete "(CITE)".

On page 8, line 24, please replace "section" with –sections–.

On page 9, line 16, please insert –min-- after "20".

On page 14, line 1, please replace "Figures" with –(Figures–.

REMARKS

If the Examiner in charge of this application believes it to be helpful, she is invited to contact the undersigned by telephone at (312) 913-0001.

Respectfully submitted,
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By: _____
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"Marked-up version of amendments:

At page 1, line 1:

This application is a divisional of U.S. patent application Serial No. 09/199,217, filed November 28, 1998, now U.S. Patent No. 6,303,324, issued October 16, 2001, which is a divisional of U.S. patent application Serial No. 08/843,008, filed April 11, 1997, now U.S. Patent No. 5,840,507, which is a continuation-in-part of U.S. provisional application Serial No. 60/041,311, filed March 19, 1997.

On page 1, line 24:

The discovery and clinical validation of markers for cancer of all types which can predict prognosis, likelihood of invasive or metastatic spread is one of the major challenges facing oncology today. In breast cancer, for example, 70% of the approximately 186,00 annual cases present as lymph node negative; however, 30% of these cases will recur after local therapy (mastectomy or "lumpectomy") (Boring *et al.*, 1992, *Clin. J. Cancer* 42: 19-38). Although adjuvant chemotherapy has been demonstrated to improve survival in node negative breast cancer patients (Mansour *et al.*, 1989, *N. Engl. J. Med.* 320: 485-490), it remains uncertain how to best identify patients whose risk of disease recurrence exceeds their risk of significant therapeutic toxicity (Osbourne, 1992, *J. Clin. Oncol.* 10: 679-682).

On page 2, line 14:

Current approaches to answer these questions stratify node negative breast cancer on the basis of primary tumor size, pathological grade, DNA S-phase fraction (SPF) and steroid hormone receptor status (Allegra *et al.*, 1979, *Cancer Treat. Rep.* 63: 1271-1277; Von Rosen *et al.*, 1989, *Breast Cancer Res. Treat.* 13: 23-32; Fischer *et al.*, 1992, *J. Natl. Cancer Inst.* 11: 152-258; Clark *et al.*, 1994, *N. Engl. J. Med.* 320: 627-633). For example, moderately and well-differentiated tumors <1 cm in size are thought to require only local excision regardless of receptor status, while such tumors from 1 to 3 cm in size that express normal levels of hormone receptor are treated with hormone therapy (Fischer *et al.*, 1993, *in* Cancer Medicine, 3d ed., Holland *et al.*, eds., Philadelphia: Lea & Febiger, pp. 1706-1774). On the other hand, patients

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with tumors larger than 2 cm that are poorly differentiated and/or hormone receptor negative are treated with adjuvant chemotherapy (Early Breast Cancer Trialistic Collaborative Group, 1992, *Lancet* 339: 1-15; The Ludwig Breast Cancer Study Group, 1989, *N. Engl. J. Med.* 320: 491-496). However, therapeutic indications are much less clearly defined for patients having moderately differentiated tumors of 1 to 3 cm in size where the hormone receptor status is borderline or unknown (Gasparini *et al.*, 1993, *J. Natl. Cancer Inst.* 85: 1206-1219). Deciding the most appropriate therapy for this group of patients, comprising about 70,000 women annually, would benefit from the development of validated prognostic analysis. Similar prognostic tools are needed in most other forms of cancer.

At page 5, line 24:

Figures 1A, 1B and 1C [Figures 1A through 1C] are histograms of the intensity of tumor marker staining *versus* tumor histology for four histological subsets described in Example 1. The p values indicate significance of the observed differences between samples of different histologies, determined using paired, one-tail t-test analysis.

At page 6, lines 4 and 9:

Figures 3A and 3B [Figure 3] is a graph of a retrospective study of patient survival of 40 breast cancer patients as described in Example 2, comparing patients having a prognostic risk index of greater than or equal to -5 (GE -5; Figure 3A) with patients having a prognostic risk index of less than -6 (LE -6; Figure 3B).

At page 7, line 16:

The methods of the invention are practiced by determining expression levels of the three preferred tumor markers (p53 nuclear accumulation, thrombospondin-1 expression and microvascularization) in a human cancer patient sample. In preferred embodiments, expression levels are determine immunohistochemically. However, expression levels can be determined using any appropriate and convenient method. For example, *in situ* polymerase chain reaction [(CITE)] and *in situ* nucleic acid hybridization methods for determining expression levels of

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TSP-1 fall within the methods of the invention. Additionally, site-specific mutation analysis, including sequence analysis or mutant allele-specific amplification of mutant p53, can be used for determining expression levels of mutant p53 in a tumor sample. Similarly, any method for detecting microvascularization, including any method of specific staining, fall within the ambit of the methods of the present invention. Detection methods are chosen appropriate for the labeling or identification of any of the three tumor markers used in the practice of the invention.

At page 8, line 24:

Tumor blocks from breast cancer patients were obtained from Western Medical Center and H. Lee Moffitt Cancer Center and examined independently by two pathologists to confirm the diagnosis for tumor type and stage. Representative [section] sections of each tumor sample were chosen on the basis of pathological examination for immunohistochemical staining. Tissue sections 5 microns in thickness were cut and prepared on slides using standard histological preparation techniques. Since paraffin sections were used, slides were first deparaffinized using Histoclear (Biogenics, California). Antigens were exposed for immunohistochemical staining by pronase digestion (for CD31 detection) and by microwave boiling (for p53 and thrombospondin 1 (TSP-1) detection) using antigen recovery solution (Biogenics). Slides were then incubated in a solution of 3% hydrogen peroxide in distilled water at room temperature for 10 min, then rinsed briefly with water. Slides were then incubated for 10 min at room temperature using 100μL goat serum as blocking buffer. Excess blocking buffer was removed from the slides by shaking, and the slides then incubated with primary antibody at room temperature for 30 min. The primary antibodies used in these assays were: antibody DO1 for p53 (obtained from Santa Cruz Biotech, Santa Cruz, CA); antibody clone 12 for TSP-1 (Immunotech, Inc., Westbrook, ME); and an endothelial cell-specific antibody reactive with the cell surface antigen CD31 for microvascularization (Dako, Carpenteria, CA). Slides were rinsed twice with phosphate buffered saline (PBS) for 5 min after primary antibody incubation.

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At page 9, line 16:

For detection of primary antibody binding, tissue sections were then incubated with biotinylated goat antimouse immunoglobulin for 20 min at room temperature in a humidified chamber (70-100% relative humidity). Slides were rinsed twice with PBS after this incubation, and then treated with a solution of peroxidase-conjugated streptavidin for 20 min at room temperature. After being rinsed again with PBS, the slides were incubated in a solution of 3,3'-diaminobenzidine for 3 min at room temperature. Slides were rinsed with PBS for 5 min, exposed to hematoxylin for 1 min, rinsed with water for 10 min, dehydrated in an ascending ethanol series, cleared with xylene, mounted and viewed by light microscopy.

At page 14, line 1:

These results demonstrated that nuclear localization of p53, decreased thrombospondin 1 expression, and increased microvascularization were significantly correlated with increased invasiveness of primary breast cancer, increased metastasis, and poorer prognosis for breast cancer patients whose tumors had these markers. To determine whether disease progression was linked not only to the incidence, but also the degree of marker expression as well, the intensity of staining of the markers as determined above by immunohistochemistry or image analysis was plotted *versus* tumor histology for the four histological subsets described above. These results are shown in Figures 1A through 1C. These results demonstrate a distinct pattern of differences in intensity and degree of expression of the three tumor markers assayed above. These results show that nuclear p53 accumulation and the number of tumor microvessels increased in both the transition from low-grade DCIS to high-grade DCIS, and also in the transition from invasive tumors without evidence of metastatic spread to invasive tumors having metastasis-positive lymph node involvement (Figures 1A and 1B). TSP-1 expression showed a significant decline in intensity between high-grade DCIS and invasive cancer prior to metastatic spread.

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